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(Enter the name and degree of Principal Investigator and any Associates)
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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The level of an mRNA depends upon its relative rates of synthesis and degradation. This is particularly important for oncoproteins and cell cycle proteins because their sustained synthesis favors cell growth rather than differentiation, a hallmark of the neoplastic phenotype. This control is exerted via a balance between the action of at least two RNA-binding proteins, AUF1 and HuR. AUF1 targets the degradation of mRNAs like the c-*myc* proto-oncogene and the cell cycle regulator cyclin D1. By contrast, HuR promotes stabilization of mRNAs. c-*myc* and cyclin D1 are particularly important, since both play causative roles in mammary tumorigenesis. Phase I of this work is to examine the effects of AUF1 and HuR expression levels on global gene expression in human breast carcinoma cells. Phase II is to assess the roles of AUF1 and HuR in cellular proliferation and tumorigenesis *in vivo*. During this funding period, we continued Phase I by transfecting expression vectors for inducible overexpression or knocked-down expression of AUF1 or HuR in human breast carcinoma cells and selecting a panel of clones. We also began identification of transcripts that are binding targets of AUF1 using mRNP immunoprecipitation (RIP) and cDNA microarray analyses of purified mRNAs.

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INTRODUCTION

The level of a messenger RNA depends not only on its rates of synthesis, processing, and transport, but also its rate of turnover. The turnover rate of an mRNA can, in turn, determine its lifetime as a template for protein synthesis. It is particularly important to understand how the levels of mRNAs encoding oncoproteins and cell cycle proteins are regulated because sustained synthesis of these gene products favors proliferation rather than differentiation, a hallmark of the neoplastic phenotype. Many cell cycle and proto-oncogene mRNAs exhibit extremely short half-lives. Their decay is controlled in part by A+U-rich elements (AREs) located in the 3'-untranslated region. Moreover, the half-lives of their mRNAs are frequently subject to regulatory control. This control is exerted via a balance between the action of at least two ARE-binding proteins, AUF1 and HuR. AUF1 targets the degradation of ARE-mRNAs such as the c-*myc* proto-oncogene and the cell cycle regulator cyclin D1. By contrast, HuR promotes stabilization of ARE-mRNAs. c-*myc* and cyclin D1 are of particular importance, since both play a causative role in mammary tumorigenesis. Our central hypothesis is that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cellular proliferation. On the other hand, HuR may act as a novel oncoprotein by stabilizing those mRNAs. To address this hypothesis, we are altering the expression of AUF1 or HuR in human breast carcinoma cells and examining the resulting effects on proliferation and tumorigenesis in a nude mouse model. There are two phases to this work: (I) to examine the effects of AUF1 and HuR expression levels on global gene expression in cultured cells; and (II) to assess the roles of AUF1 and HuR in proliferation and tumorigenesis *in vivo*. For Phase I we have introduced into human breast carcinoma cells expression vectors that will permit either overexpression or knocked-down expression of AUF1 or HuR. We will classify clones by their expression levels of AUF1 or HuR (e.g. low, medium, or high) and then profile the resulting effects on global gene expression using a novel cDNA microarray strategy. Genes involved in the cell cycle, metastasis and invasion, and angiogenesis are of particular interest, as many of these contain AREs. In Phase II, we will characterize clones for both their growth phenotypes and their ability to induce tumors as xenographs on nude mice. Our prediction is that high AUF1 levels will reduce the expression of ARE-mRNAs important for the cell cycle, invasion, metastasis, and angiogenesis. This will likely lower the efficacy of engineered cell lines to induce tumors. By contrast, high HuR levels will likely increase expression of these genes and lead to increased tumorigenic efficiency.

BODY

The approved SOW is as follows:

Task 1. To examine the effects of AUF1 and HuR expression levels on gene expression in cultured breast carcinoma cells (Months 1-24):

a. Construct Tet-Off plasmids for overexpression of AUF1 and HuR (Months 1-4).
b. Construct plasmids for RNA interference (RNAi)-based knockdown of AUF1 and HuR (Months 1-4).
c. Transfect plasmids into breast carcinoma Tet-Off cells and select individual clones (Months 4-24).
d. Characterize clones for AUF1 and HuR expression levels to define those with low, medium, and high levels of AUF1/HuR expression (Months 6-24).
e. Using RNA from engineered cell lines, perform DNA microarray analyses of genes affected by altered AUF1/HuR expression, in particular, those involved in the cell cycle, invasion, metastasis, and angiogenesis (Months 6-24).
f. Identify those transcripts that are direct binding targets of AUF1 and HuR (Months 6-24).

Task 2. To assess the roles of AUF1/HuR in cell growth and tumorigenesis (Months 6-36):

a. Characterize the growth phenotypes of engineered cell lines obtained from Task 1d (Months 6-24).
b. Characterize the cell cycle distribution of these cell lines by flow cytometry (Months 6-24).
c. Introduce clones onto nude mice and score tumor formation as a function of AUF1/HuR expression levels and growth/cell cycle phenotypes (Months 6-36).

For Task 1-a, -b, and –c, we have completed preparation of tetracycline-regulated expression constructs for $p37^{AUF1}$ (plasmid pTRE/p37), $p40^{AUF1}$ (plasmid pTREaI/p40), and HuR (plasmid pTREaI/HuR) as described in last year's progress report. These plasmids were transfected into MCF-7 Tet Off cells (human breast carcinoma) and drug selection yielded cell clones for each construct. Individual clones were picked from tissue culture plates. Since individual clones exhibit unique growth rates, some have expanded to the point where they could be frozen. At the same time, whole cell lysates were prepared from these clones for western blot analyses. Others are growing slowly and have not yet reached cell numbers sufficient for freezing and lysate preparation. Table I (next page) describes the number of clones we have for each transfected construct and the status of each clone. We will begin shortly the western blot analyses of the lysates we already have (Task 1-d). As we accrue more lysates, we will analyze these as well.

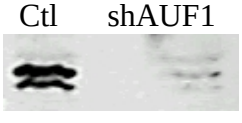

Figure 1. See text for details

Also related to Tasks 1-a, -b, and -c, we completed preparation of tetracycline-regulated expression constructs for short hairpin RNAs (shRNAs) designed to knockdown expression of AUF1 (all isoforms together) and HuR. As a prelude to transfecting these into MCF-7 Tet Off cells and selecting clones, we performed transient transfections with each plasmid to insure that the shRNA would indeed knockdown protein expression. Figure 1 is a western blot of such a pilot experiment with the

AUF1 shRNA vector. Clearly, this shRNA is very effective for RNAi-induced knockdown of AUF1 [Figure 1, compare lane 2 (AUF1 shRNA) to lane 1 (control)]. However, Figure 2 shows that the shRNA for HuR is not effective [compare lane 2 (HuR shRNA) to lane 1 (control)]. Thus, we will have to test other shRNAs for HuR. Nonetheless, the excellent results with the shRNA for AUF1 encouraged us to transfect it into MCF-7 Tet Off cells and select individual drug-resistant clones. Table I describes the status of each clone for the AUF1 shRNA vector. Once we accrue more lysates of clones, we will begin western blot analyses to characterize them for AUF1 knockdown (Task 1-d). Once we characterize all of our clones for AUF1/HuR knockdown or overexpression, we can begin Task 1-e.

Table I. Status of clones transfected with overexpression and shRNA vectors

Vector	Number of isolated clones	Number of expanded clones	Number of frozen clones
pS U6/tetO/hyg/CTL	6	6 in progress	-
pS U6/tetO/hyg/AUF1	20	14	8
pTRE/p37^{AUF1}	21	7 in progress	-
pTREaI/p40^{AUF1}	21	18 in progress	-
pTREaI/HuR	18	1 in progress	-

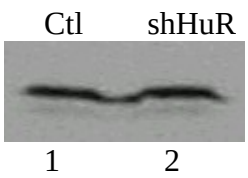

Figure 2. See text for details.

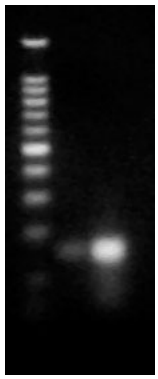

Figure 4. See text for details

Task 1-f does not require either the AUF1/HuR knockdown or over-expression cell lines. It is sufficient to identify the RNA-binding targets of AUF1 and HuR in parental MCF-7 Tet Off cells. Thus, we worked hard this year to establish the conditions for mRNP immuno-precipitation (RIP) assays. This assay utilizes an antibody to an RNA-binding protein to immuno-precipitate mRNPs associated with that protein. We immuno-precipitated mRNPs with our AUF1 antibody or preimmune serum (as a control) and purified the RNAs in the precipitates. To verify that we purified AUF1 target mRNAs, we performed quantitative RT-PCR (qPCR) using primers and a fluorescent probe specific for c-*myc* mRNA, a known AUF1 target. Figure 3 (in Appendices, page 9) is a plot of fluorescence signal *versus* cycle number. The C_t with the preimmune sample is 32.54 and the C_t for the AUF1 antibody sample is 29.24. The ΔC_t represents an ~8-fold difference and demonstrates that the AUF1 antibody can immunopurify c-*myc* mRNA. The products of the qPCR reaction were fractionated in an agarose gel to verify that the PCR product was the predicted size. Lane 1 in Figure 4 contains 100-bp increment size markers. (The brightest is 500 bp.) The gel indicates that the PCR products in Figure 4, lanes 2 and 3, are ~150 bp, the predicted size. Note also that the intensity in lane 3 (the anti-AUF1 sample) is much higher than lane 2 (the control sample), consistent with the qPCR results. We have since sent these RNA samples to our in-house cDNA

Microarray Facility for identification of purified RNAs. We are still (impatiently) awaiting the results.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of vectors for overexpression of $p37^{AUF1}$, $p40^{AUF1}$, and HuR and knockdown of AUF1. (The shRNA vector for HuR is not effective for knockdown.)
- Transfection of selected plasmids into MCF7-Tet Off breast carcinoma cells and expansion of drug-selected clones. Characterization is in progress.
- Immunopurification of AUF1 target mRNAs and cDNA microarray analyses for their identification (awaiting results).

REPORTABLE OUTCOMES

We are still in the process of building the tools that will allow us to assess the biological effects of reengineering expression of the key ARE-binding proteins AUF1 and HuR in human breast carcinoma cells. We have, and are still selecting, clones for elevated or reduced expression of AUF1 or HuR. These cell lines do not, per se, constitute a reportable outcome (i.e., a peer-reviewed publication). The results of the cDNA microarray to identify AUF1 target mRNAs will be important, but only in the context of experiments utilizing the clones mentioned above.

CONCLUSIONS

Our central hypothesis is that AUF1 may act as a novel tumor suppressor by limiting expression of genes that promote cell growth. On the other hand, HuR may act as a novel oncoprotein by stabilizing those mRNAs. Our approach is to alter the expression of AUF1 or HuR in human breast carcinoma cells and examine the resulting effects on cell growth and tumorigenesis in a nude mouse model. Specifically, our work has two phases: (I) to examine the effects of AUF1 and HuR expression levels on gene expression in cultured cells; and (II) to assess the roles of AUF1 and HuR in cellular proliferation and tumorigenesis *in vivo*. We are currently focused on Phase I. This phase requires us to prepare expression vectors that will permit either overexpression of knocked-down expression of these two proteins. We have completed most of the vectors, transfected these into human breast carcinoma cells, and have obtained a panel of drug-selected clones. The shRNA vector for HuR knockdown has not worked, probably due to poor expression/processing of the shRNA. Interestingly, we used the same sequence shown to be effective as a short interfering RNA (siRNA) in the design of our shRNA. It's likely that the loop sequence in the shRNA is not processed well by Dicer in cells. Thus, we will design a series of vectors with different loop sequences. Additionally, we will design vectors with shRNAs antisense to different regions of the HuR mRNA. A microRNA to HuR is a possibility as well. Open Bioystems has a website (http://www.openbiosystems.com) of microRNAs that are validated for knockdowns; they list several for HuR, which we can try.

The identification of the AUF1 target mRNAs in breast carcinoma cells (already in progress) will be exciting. We will have the opportunity to combine this knowledge with the results of the AUF1 or HuR knockdown/overexpression experiments to ascertain which gene expression networks are

controlled by AUF1 (and HuR, at some point) and how altered AUF1/HuR expression impacts them and tumor formation/progression as well.

To place our work in perspective, the discovery of AREs in 1986 and the subsequent identification of the ARE-binding proteins that control ARE-mRNA decay have led to the realization that many genes that play active roles in oncogenesis are regulated via their AREs. What has unfortunately received scant attention is the idea that the regulators of these important ARE-mRNAs might themselves possess oncogenic or tumor suppressor activities. Our work is designed to address this idea and provide two potential contributions. (1) They could have a major impact on our thinking about the pathways by which normal cells become transformed. This is particularly true in breast cancer, since c-*myc* and cyclin D1, two AUF1-regulated mRNAs, are frequently overexpressed. (2) Our studies could spur development of a new generation of pharmaceuticals designed to target an ARE-binding protein such as AUF1 or HuR. This strategy would permit control of large networks of ARE-mRNAs by simply acting on a single target ARE-binding protein.

REFERENCES

None

APPENDICES

Figure 3, next page.

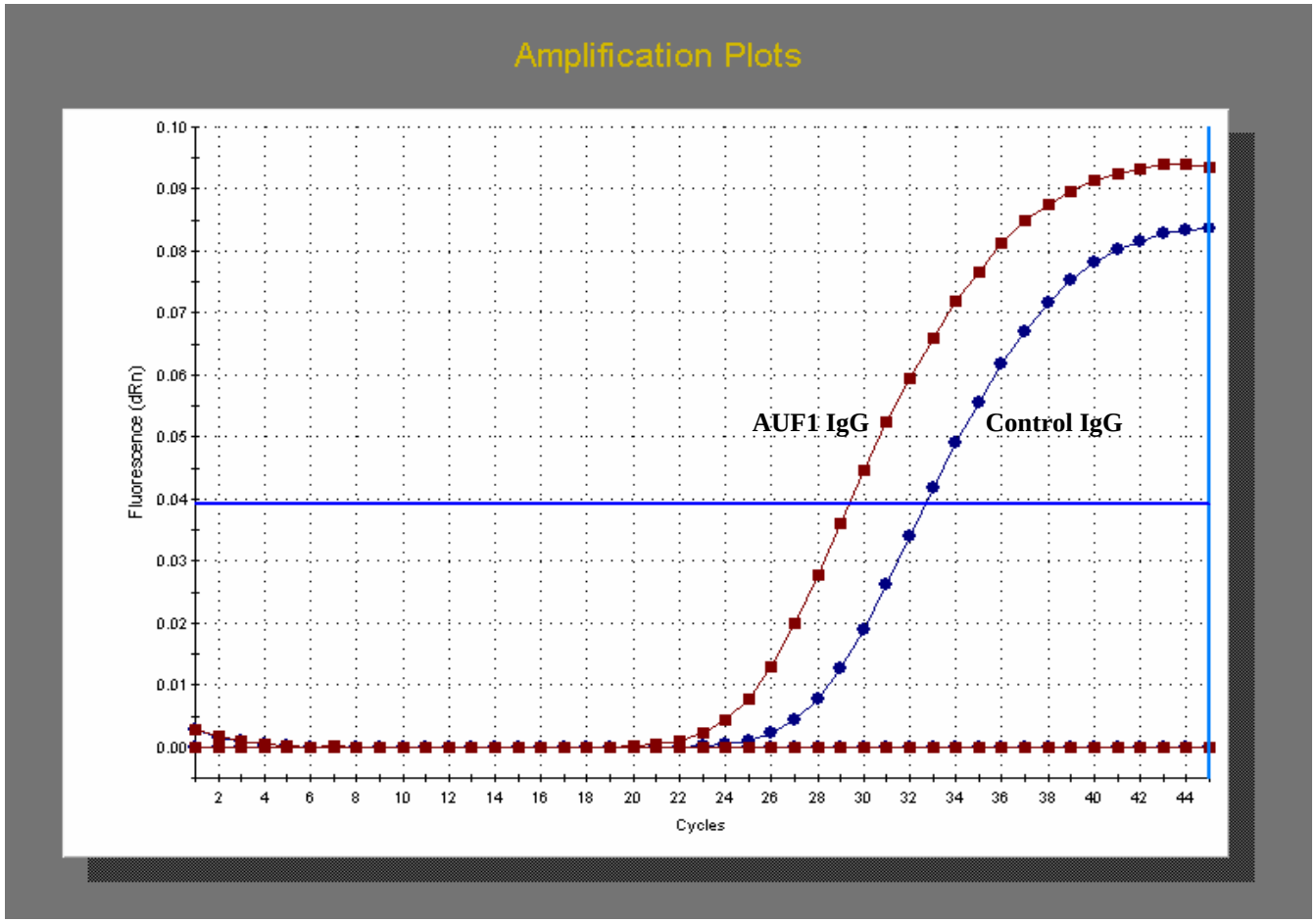

Figure 3. Amplification plots. mRNPs were immunoprecipiated with either control IgG or anti-AUF1 IgG. RNA was extracted and analyzed for c-*myc* mRNA by quantitative RT-PCR. The amplification plots are shown. The C_t for control IgG is 32.54 and the C_t for anti-AUF1 IgG is 29.24, indicating an ~8-fold enrichment of c-*myc* mRNA over the control.